PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number:
A01N 63/00	A1	WO 91/02459
		(43) International Publication Date: 7 March 1991 (07.03.91)

(21) International Application Number: PCT/US90/04714

(22) International Filing Date: 20 August 1990 (20.08.90)

(30) Priority data:
395,625 18 August 1989 (18.08.89) US

(71) Applicant: UNIVERSITY OF FLORIDA [US/US]; 223 Grinter Hall, Gainesville, FL 32611 (US).

(72) Inventors: NARAYANAN, Komaratchi, R. ; 13861 S.W. 275 Terrace, Homestead, FL 33032 (US). McMILLAN, Robert, T., Jr. ; 26100 S.W. 197th Avenue, Homestead, FL 33031 (US).

(74) Agents: SALIWANCHIK, David et al.; Saliwanchik & Saliwanchik, 2421 N.W. 41st Street, Suite A-1, Gainesville, FL 32606 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NOVEL METHODS AND COMPOSITIONS FOR THE CONTROL OF FUNGI AND BACTERIA

(57) Abstract

Proteinases, especially sulfhydryl proteinases such as papain, bromelain, and ficin, are effective in controlling pathogenic bacterial and fungi. When applied to the surface of plants, the proteinases of the subject invention inhibit the growth of fungi and bacteria. These compounds are particularly useful with plants because they are not phytotoxic. In addition to their use on plants, the compounds of the subject invention may also be used on any other surface in need of a bactericidal or fungicidal agent. Also, the proteinases of the subject invention can be used as preservatives or sterilants of materials susceptible to microbial contamination.

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LU	Luxembourg	TG	Togo
				US	United States of America

DESCRIPTION

NOVEL METHODS AND COMPOSITIONS FOR THE CONTROL OF FUNGI AND BACTERIA

Background of the Invention

One of the major problems facing the agriculture industry is the control of insect pests and disease. Of the many diseases which affect plants, a great number are of bacterial or fungal origin. Fungal and bacterial plant diseases can be especially problematic in hot, humid climates such as that which exists in Florida and other southern areas.

An example of an important vegetable disease caused by bacteria is bacterial leaf spot caused by Xanthomonas campestris pv vesicatoria. This pathogen causes widespread disease affecting Florida tomatoes (Pohronezny, K., V.H. Waddill, D.J. Schuster, and R.M. Sonoda [1986] Plant Dis. 70:96-102). Yield losses of up to 30% due to bacterial leaf spot have been reported (Pohronezny, K., and R.B. Volin [1983] HortScience 18:69-70).

Control efforts to date have focused on the identification of chemical control agents. At the present time, registered commercial pesticide sprays do not provide an acceptable level of control of bacterial leaf spot. A combination of copper and mancozeb provides a limited amount of control, but it is primarily effective only at low disease pressure.

In addition to being largely ineffective, current efforts to control fungi and bacteria present the further disadvantages of polluting the environment and creating potential health hazards to agricultural workers and to consumers, who may be exposed directly to the chemicals during application or to residues which can remain on the crops. Additional problems associated with traditional chemical pesticides include the development of resistance in target species,

detrimental effects of these chemicals on non-target species, and phytotoxic reactions by treated plants.

Because of the problems associated with the use of traditional fungicides and bactericides, safer and more effective methods of control for bacteria and fungi are clearly needed. This is true, not only for use on tomato crops, but also for other crop plants and for non-agricultural uses as well.

Brief Summary of the Invention

The subject invention concerns the discovery that proteinases, especially sulfhydryl proteinases such as papain, bromelain, and ficin, are effective in controlling pathogenic bacteria and fungi. Xanthomonas campestris pv vesicatoria, Fusarium oxysporum F. sp. lycopersici biotype 3, Verticillium albo-atrum biotype 2, and other bacteria and fungi were found to be effectively inhibited by several sulfhydryl proteinases including papain.

Detailed Description of the Invention

The role of proteinases and proteinase inhibitors in plants is not thoroughly understood. Some of the roles assigned to proteinases include (a) storage protein breakdown, (b) intracellular protein turn over, (c) role in development including senescence, and (d) proteolytic modification of proteins (Ryan and Walker-Simmons, 1981).

It has been discovered that some plants such as Ipomea batatas, Lycopersicon peruvianum, Solanum macranthum, kudzu, and pigeon pea, which have tolerance to many bacterial and fungal diseases, also contain high levels of endogenous proteinase activity. In order to determine whether there was any casual relationship between the presence of proteinases and the observed resistance to certain pathogens, a number of experiments have been conducted to assess the efficacy of proteinases in controlling bacteria and fungi. These

experiments have been conducted in vitro and in the field. In vitro tests demonstrated that sulfhydryl proteinases were very effective in inhibiting the growth of both fungi and bacteria. For example, the sulfhydryl proteinase known as papain significantly reduced the incidence and severity of bacterial leaf spot in detached leaves of tomato. Furthermore, it was found that papain reduced the incidence of bacterial leaf spot in whole plants in growth chamber experiments.

In further experiments, papain and two other proteinases were found to be highly effective in inhibiting the growth of fungi. The proteinases were found to be effective against Fusarium and Verticillium.

Importantly, the proteinases of the subject invention were found to actually increase marketable yield obtained from tomato plants treated with proteinase as compared to yields obtained from plants treated with bactericides comprising copper. This increase in marketable yield may reflect the lack of phytotoxicity of the proteinases. These experiments also show that the compounds of the subject invention are not degraded too rapidly to exert their beneficial effects. However, because these compounds are proteins, they should be environmentally safe because they will not persist in the environment.

The proteinases of the subject invention are effective antifungal and antibacterial agents when they are simply applied to the surface of a plant, such as on the leaves of the plant. To facilitate this application, the proteinases may be mixed with an appropriate liquid or powder agricultural carrier. Suitable liquid diluents or carriers for use in the conduct of this invention include water, petroleum distillates, or other liquid carriers, with or without various dissolved salts and surface active emulsifying and dispersing agents. These liquid compositions may be prepared by dissolving or dispersing a bactericidal or fungicidal amount of a proteinase in the appropriate biologically compatible diluent or carrier.

Solid compositions may be prepared by dispersing the desired proteinases in or on an appropriately divided carrier such as clay, talc, bentonite, diatomaceous earth, fuller's earth, and the like. When such formulations are used as wettable powders, biologically compatible dispersing agents such as liquosulfonates and various non-ionic, anionic, amphoteric, or cationic dispersing and emulsifying agents can be used.

Whether a liquid or solid carrier is used, the proteinases may be combined with other fungicidal, bactericidal, or pesticidal compositions, as well as with fertilizers and plant growth regulators, so long as these other compositions do not denature the proteinase or otherwise inhibit its activity. The proteinases may also be encapsulated or otherwise formulated so as to increase their longevity in the field. Techniques for such encapsulation are known in the art.

When applied to the surface of plants, the proteinases of the subject invention inhibit the growth of fungi and bacteria. These compounds are particularly useful with plants because they are not phytotoxic. In addition to their use on plants, the compounds of the subject invention may also be used on any other surface in need of a bactericidal or fungicidal agent. Also, the proteinases of the subject invention can be used as preservatives or sterilants of materials susceptible to microbial contamination.

Materials and Methods

Plant Material. All the plants used in this study were obtained from the Tropical Research and Education Center of the University of Florida at Homestead. Seeds of Lycopersicon esculentum L. cv Flora-Dade were from the germ plasm collections and the Tropical Research and Education Center.

Chemicals. Chemicals were purchased from Sigma Chemical Company and Fisher Scientific. All chemicals were of the highest purity available.

In vitro Inhibition Studies. Purified papain was either dialyzed (to remove preservatives such as thymol) against 10 mM phosphate buffer, pH 7, containing dithiothreitol or used directly after dilution with buffer containing dithiothreitol. Spores of Fusarium oxysporum Schlecht biotype 3 were treated for 1 hour with 0, 10 mg/ml dialyzed papain, or 10 mg/ml of undialyzed papain at room temperature. After 1 hour, 100 ul of spore suspension was spread on plates. After 2-3 days, the plates were evaluated for growth of Fusarium.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 – Assay of Bacterial Leaf Spot in Detached Leaves

Fully expanded leaves from 5-week old Lycopersicon esculentum L. cv Flora-Dade plants grown in the greenhouse were immersed in deionized water with the petioles submerged in water. With a brush, 0, 0.23, 2.3, or 23 units/ml of papain (2.3 units/mg of papain) was applied to the detached leaves of tomato. This was immediately followed by application of Xanthomonas campestris pv vesicatoria on the surface of all leaves. The leaves, in individual test tubes, were kept in the greenhouse for 2 weeks prior to evaluation for the incidence and severity of bacterial leaf spot. There were three replications consisting of 4 leaves for each treatment for each replication. The results of these experiments are shown in Table 1.

Table 1. Effect of thiol proteinase on severity of bacterial leaf spot in detached leaves of tomato

Concentration of proteinase (units/ml)	# spots/leaf	% reduction in severity of disease
0	49.3±4.2	0
0.23	10.3±3.7	79.1
2.3	4.1±2.1	91.7
23.0	1.0±0.3	98.0

A strong protective effect of papain against bacterial leaf spot was observed. Both the incidence and severity of bacterial leaf spot were reduced by treatment with papain. These experiments were successfully repeated on whole plants in the greenhouse. For the greenhouse experiments, the plants used were Lycopersicon esculentum cv 'Horizon.' The plants were 2 weeks old when they were infected with Xanthomonas campestris pv vesicatoria after spraying with papain at appropriate concentrations. There were 20 plants for each treatment with 2 replications. The plants were kept in a hot dew chamber for 2-3 days prior to evaluation. The results of this study are shown below in Table 2. Significant reductions in the number of lesions on these plants were seen when proteinase was applied.

Table 2. Effect of thiol proteinase on the incidence and severity of bacterial leaf spot in whole plants.

Concentration of proteinase mg/ml	% plants with symptoms	# of lesions/cm^2
0	80	4.00
	75	3.10
1	20	0.40
	15	0.25
10	15	0.20
	10	0.10

Example 2

Field experiments were conducted wherein papain was compared against copper/mancozeb applications in grower fields. The results of these field experiments are shown in Table 3. The field experiments were designed to assess the marketable yield which could be obtained from plants which were treated with proteinase as compared to plants which were treated with a copper and mancozeb mixture. The copper treatment is the form of control which is most widely practiced at the present time. The results of a test such as this are particularly illuminating because it indicates how well the control agent works under actual field conditions.

Table 3.

Replication	Total marketable yield (lbs.)		
	Copper	Protease	% difference using protease
1	1548	1781	+15.0
2	1480	1648	+11.4
3	1420	1717	+20.9
4	1247	1858	+49.0
Average	1424	1751	+23.0

As can be seen in Table 3, the marketable yield obtained from plants treated with proteinase ranged from 11% to nearly 50% higher than the yield obtained after treatment with the copper mixture. This shows that proteinase is not only effective in inhibiting bacterial leaf spot, but also that plants treated with proteinase produce a greater marketable yield than plants treated with other compositions which control bacterial leaf spot. This enhanced yield may be due to the low phytotoxicity of proteinases as compared to the copper composition.

The success of these field experiments also clearly demonstrates that these proteins retained sufficient activity to exert their beneficial effects despite exposure to sunlight, wind, and rain. Advantageously, however, these compositions will not persist in the environment in the manner that many other pesticides do. Therefore, these control agents are both effect and environmentally safe.

Example 3 – Inhibition of *Fusarium oxysporum* Biotype 3 by Sulfhydryl Proteinases

Purified papain from Sigma Chemical Company (#P 3125) was either dialyzed (to remove preservatives such as thymol) against 10 mM, pH 7 phosphate buffer containing 1 mM dithiothreitol (DTT) or used directly after dilution with buffer containing DTT. Spores of Fusarium oxysporum biotype 3 were treated for 1 hour with 0, 10 mg/ml dialyzed papain, or 10 mg/ml undialyzed papain at room temperature. After 1 hour, 100 ul of spore suspension was spread on plates. After 2-3 days, the plates were evaluated for growth of Fusarium. Table 4 shows the effect of papain on Fusarium oxysporum biotype 3. The treatment of spores for 1 hour completely inhibited the growth of spores.

Other sulfhydryl proteinases were also tested for their effectiveness in inhibiting the formation of Fusarium oxysporum colonies. As can be seen in Table 4, other sulfhydryl proteinases were also very effective in preventing the formation of colonies. In the case of Bromelain, it was important that the proteinase was dialyzed. Dialyzation removes impurities, such as proteinase inhibitors which can adversely affect the performance of the proteinase.

Table 4. Effect of different sulfhydryl proteinases on Fusarium oxysporum

Treatment	Blank	Bromelain		Ficin		Papain	
		1	2	1	2	1	2
Control	0*	0	0	0	0	0	0
Fusarium	155	81.3	0.7	0	0	0	0

*no. of colonies; 1 = nondialyzed material; 2 = dialyzed material

Claims

1. A method for inhibiting fungal or bacterial growth, said method comprising the application to a surface or material in need of protection from fungi or bacteria, a fungicidally or bactericidally effective amount of a composition comprising one or more proteinases.

2. A method for inhibiting fungal or bacterial growth on plants, said method comprising the application to the surface or situ of said plant a fungicidally or bactericidally effective amount of a composition comprising one or more proteinases.

3. The method, according to claim 2, wherein said proteinase is found in plants.

4. The method, according to claim 2, wherein said proteinase is produced by the plant but which does not play a significant role in specific biological activities.

5. The method, according to claim 2, wherein said proteinase is a sulfhydryl proteinase.

6. The method, according to claim 2, wherein said proteinase is selected from the group consisting of papain, ficin, and bromelain.

7. The method, according to claim 2, wherein said proteinase is papain.

8. The method, according to claim 2, wherein said plant is a vegetable crop plant.

9. The method, according to claim 2, wherein said plant is a tomato.

10. The method, according to claim 2, wherein said proteinase has been dialyzed.

11. The method, according to claim 2, wherein said proteinase is mixed with an agriculturally acceptable carrier or diluent.

12. The method, according to claim 2, wherein said proteinase is mixed with one or more ingredients selected from the group consisting of fungicides, bactericides, insecticides, fertilizers, and plant growth regulators.

13. The method, according to claim 2, wherein said proteinase has been encapsulated or otherwise modified to prolong its activity.

14. The method, according to claim 2, wherein said method is used to inhibit the growth of Fusarium oxysporum.

15. The method, according to claim 2, wherein said method is used to control Xanthomonas campestris.

16. A method for controlling fungal or bacterial plant pathogens, said method comprising the application to a plant surface an effective amount of a composition comprising a proteinase.

17. The method, according to claim 16, wherein said proteinase is a sulfhydryl proteinase.

18. The method, according to claim 16, wherein said plant is a tomato plant.

19. The method, according to claim 16, wherein said pathogen is Xanthomonas campestris.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/04714

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 01 N 63/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0184288 (FBC LTD) 11 June 1986 see page 3, line 25 - page 6, line 7; example 3; claims --	1,2,5,6,8, 9,11,16-18
P, X	WO, A, 90/03732 (NOVO-NORDISK A/S) 19 April 1990 see page 5, lines 1-38; examples 1-5; claims --	1,2,5,8,11, 12,16,17
X	Derwent Central Patents Index, Basic Abstracts Journal, section C, AGDOC, week A18, & JP, A, 53029911 (IWATA) 20 March 1978 see the abstract --	1-7

./.

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	24. 01. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	Chemical Abstracts, vol. 97, no. 13, 27 September 1982, (Columbus, Ohio, US), see page 202, abstract 105601c, & JP, A, 82 85307 (ASAHI DENKA KOGYO K.K.) 28 May 1982 --	1-5,12,16, 17
X	Derwent Central Patents Index, Basic Abstracts Journal, section C, AGDOC, week B29, 17 September 1979,Derwent Publications Ltd, (London, GB), & JP, A, 54073182 (MITSUI PETROCHEM. IND. K.K.) 12 June 1979 see the abstract --	1,2,8,16
X	Chemical Abstracts, vol. 87, no. 11, 12 September 1977, (Columbus, Ohio, US), see page 160, abstract 79669c, & JP, A, 77 12245 (INSTITUTE OF PHYSICAL AND CHEMICAL RESEARCH) 6 April 1977 ----------	1,2,8,16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9004714 SA 40289

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 15/01/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0184288	11-06-86	JP-A- 61143309	01-07-86
WO-A- 9003732	19-04-90	AU-A- 5102990	01-05-90